Separation of supercoiled from open circular forms of plasmid DNA, and biological activity detection

Huangjin Li · Huaben Bo · Jinquan Wang ·
Hongwei Shao · Shulin Huang

Received: 7 September 2010 / Accepted: 13 November 2010 / Published online: 1 December 2010
© Springer Science+Business Media B.V. 2010

Abstract To establish a cost-effective purification process for the large-scale production of plasmid DNA for gene therapy and DNA vaccination, a single anion-exchange chromatography (AEC) step was employed to purify supercoiled plasmid DNA (sc pDNA) from other isoforms and *Escherichia coli* impurities present in a clarified lysate. Two different size and conformation plasmids were used as model targets, and showed similar elution behavior in this chromatographic operation, in which sc pDNA was effectively separated from open circle plasmid DNA (oc pDNA) in a salt gradient. The process delivered high-purity pDNA of homogeneity of $95 \pm 1.1\%$ and almost undetectable levels of endotoxins, genomic DNA, RNA and protein, at a yield of $65 \pm 8\%$. Furthermore, the transfection efficiency ($29 \pm 0.4\%$) was significantly higher than that ($20 \pm 0.1\%$) of a pDNA control. The present study confirms the possibility of using a single AEC step to purify sc pDNA from other isoforms and host contaminants present in a clarified *E. coli* lysate.

Keywords Anion-exchange chromatography · Supercoiled plasmid DNA · Open circular plasmid DNA · Gene therapy

Introduction

The recent developments in nonviral gene therapy and DNA vaccination have fostered the development of efficient plasmid DNA (pDNA) purification process (Diogo et al. 2005; Bencina et al. 2004). The number of naked/plasmid DNA studies has dramatically increased accounting for over 17.7% of all gene therapy clinical trials (www.wiley.co.uk/genmed/clinical, 2009), just behind adenoviruses (23.9%) and retroviruses (20.8%). In comparison to viral vectors, non-viral ones like naked pDNA are considered to be safer, but they are less effective. Several lines of evidence indicate that high supercoiled levels are required for eliciting an effective immune response and ultimately, protection from infectious challenge (Cupillard et al. 2005; Pillai et al. 2008). So the separation of supercoiled (sc) and open circular (oc) isoforms is one of the key steps in the large-scale purification of pDNA vectors for gene therapy and DNA vaccination.

Plasmids are usually produced in an *Escherichia coli* host by fermentation and are purified by a sequence of operations (Prazeres et al. 1998). Although *E. coli* produces mainly the more compact sc pDNA isoform (Prazeres et al. 1998), oc, linear and denatured pDNA isoforms are usually present due to conformational changes that occur within the bacterial host or during processing of biomass (Schleef and Schmidt 2004). For instance, an intact and undamaged form of sc pDNA may give rise to oc

H. Li · H. Bo · J. Wang · H. Shao · S. Huang (✉)
School of Life Science and Bio-Pharmaceutical,
Guangdong Pharmaceutical University, 510006
Guangzhou, China
e-mail: ben1702@126.com

or linear isoforms by random cleavage (e.g., enzymatic, chemical) of one or two opposing strands, respectively (Iuliano et al. 2002). Furthermore, irreversibly denatured covalently closed pDNA isoforms are typically generated during alkaline lysis if the pH is not kept below 12.5 (Prazeres et al. 1998). Whatever the case, the resulting isoforms are likely to be less efficient in transferring gene expression if cleavage or strand melting disrupts promoter or gene coding regions (Schleef and Schmidt 2004). For this reason, regulatory agencies specify that the homogeneity of a therapeutic pDNA product, expressed as percentage of the sc isoform, should be higher than 90% (Schleef and Schmidt 2004).

There are different chromatographic techniques implemented for pDNA purification, such as size-exclusion chromatography (SEC) (Horn et al. 1995; Ferreira et al. 1997), anion-exchange chromatography (AEC) (Prazeres et al. 1998; Hines et al. 1992), hydrophobic interaction chromatography (HIC) (Gurunathan et al. 2000; Diogo et al. 2000, 2001, 2003) or affinity chromatography (AC) (Wils et al. 1997; Schluep and Cooney 1998; Sandberg et al. 2004). These chromatographic methods work well in removing host impurities (protein, RNA, endotoxins, genomic DNA etc.), but they can not separate sc pDNA from oc pDNA just by a single chromatographic step.

The aim of our work was to investigate the possibility of removing host impurities and separating sc from oc pDNA conformation just by a single chromatographic step. To evaluate the novel method, two different size plasmids pCDNA3.1-GFP (7,200 bp) and pT-survivin 2B (3,500 bp) were used as model target.

Materials and methods

Materials

The *E. coli* host strain DH5 α containing pCDNA3.1-GFP (7,200 bp), a plasmid encoding GFP, and the *E. coli* host strain DH5 α containing pT-survivin 2B (3,500 bp), a plasmid encoding survivin 2B, were constructed by the Institute of Biopharmacology of Guangdong Pharmaceutical University.

DEAE Sepharose Fast Flow, Sephacryl S-500 High Resolution, a XK 16/40 column, AKTA Basic system and Frac-920 fraction collector were provided

by Amersham Bioscience. Beckman Avanti J-25 centrifuge was purchased from Beckman Co. Tianli gel scanner was the product of Tianli Biotech.

Lipofectamine 2000 and RPMI 1640 were purchased from Invitrogen, fetal bovine serum from TBD, Luria broth (LB) from Sigma and agarose from FMC. All salts used were of analytical grade.

Methods

Bacterial culture

E. coli DH5 α harboring pCDNA3.1-GFP and pT-survivin 2B were grown overnight, at 37 °C, in shake flasks containing 500 mL of LB medium supplemented with 100 μ g/mL Ampicillin, at 200 rpm. Cells were harvested by centrifugation at late log phase.

Lysis and primary isolation

Cells were lysed using the alkaline method. Cells were centrifuged at 5,000g for 15 min at 4 °C by Beckman Avanti J-25 centrifuge. Supernatants were discarded and pellet resuspended in 25 mL of 50 mM glucose, 10 mM ethylene-diamine tetraacetic acid (EDTA), 25 mM Tris/HCl, pH 8.0. Lysis was performed on ice by adding 25 mL of a prechilled 200 mM NaOH, 1% (w/v) sodium dodecylsulfate solution (SDS). Cellular debris, genomic DNA, and proteins were precipitated by gently adding 20 mL of prechilled (on ice) 3 M potassium acetate, pH 5.0. The precipitate was removed with centrifugation at 12,000g (20 min, 4 °C).

The neutralized lysate was precipitated by slowly adding solid CaCl₂ to a final concentration of 0.4 M with constant mixing. Suspension was left at 4 °C for 15 min, then centrifuged at 12,000g for 20 min at 4 °C. Supernatant was filtered through 0.45 μ m filter and decreased ionic strength (around 34 mS/cm) by adding dH₂O, then loaded directly on the AEC columns.

Anion-exchange chromatography (AEC)

An XK 16/40 (40 cm $\times$ 1.6 cm) column was packed according to the manufacturer's instructions with DEAE Sepharose Fast Flow, a weak anion exchanger. Chromatography was performed with AKTA Purifier

system. The column was equilibrated with 4 column volumes (CV) of loading buffer (0.3 M sodium chloride in 10 mM Tris/HCl, pH 8.0) at a flow rate of 7 mL min⁻¹. The plasmid sample was loaded on the column at a flow rate of 7 mL min⁻¹. The column was washed with 2 CV of loading buffer. A linear gradient from 0.3 to 0.5 M was carried out with sodium chloride solution in 10 M Tris/HCl, pH 8.0 in 10 CV at a flow rate of 7 mL min⁻¹. Absorbance was monitored at 254 nm. Plasmid-containing fractions were collected and kept for further analysis. The column was cleaned with 2 CV of 1 M NaOH.

Desalting

Plasmid samples were desalted prior to protein assay and electrophoresis to prevent the interference of the salt with the analysis. Sephacryl S-500 High Resolution column was used with 10 mM Tris/HCl, pH 8.0, as running buffer.

Agarose gel electrophoresis

Samples from AEC were analyzed by horizontal electrophoresis using 0.8% agarose gels run at 100 V, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0), and stained with ethidium bromide (0.5 µg mL⁻¹).

Homogeneity analysis

The homogeneity of pDNA was analyzed by gel scanning with Tianli gel scanner according to the manufacturer's instructions. The homogeneity was expressed as the percentage of the sc pDNA in total pDNA.

Protein analysis

Protein concentration was determined using the BCA (bicinichonic acid) assay from Biotek (China). Twenty microliter samples were pipetted into the wells of a microplate and 200 µl of the working reagent was added to each well. After homogenization, the microplate was incubated at 37 °C for 30 min. Absorbance was measured at 570 nm in a microplate reader. A calibration curve was made using bovine serum albumin as the protein standard.

Endotoxin analysis

Endotoxins were quantitatively assayed by the kinetic-QCL Limulus amoebocyte lysate assay kit from Biowhittaker (Walkersville, MD), according to the manufacture's instructions. The detection level for the method used was 0.010 EU/mL.

Purity analysis

Purity considered as the relative DNA/protein content was estimated by spectrophotometry, and expressed as the ratio of absorbance at 260 nm and at 280 nm respectively (A_{260}/A_{280}).

Biological activity detection

The plasmid's biological activity was detected by liposomes transfection experiment with 7,402 cell. 4×10^5 cells were plated in 2 mL of growth medium without antibiotics and cultured overnight to a confluence of 90–95%. 4 µg plasmid DNA and appropriate amount Lipofectamine 2000 were, respectively diluted in 250 µl RPMI 1640 medium without serum, incubated for 5 min at room temperature, combined by gently mixing, then incubated for 20 min at room temperature. All of the 500 µl complexes were added to each well containing cells and medium, mixed gently by rocking the plate back and forth, then incubated at 37 °C in a CO₂ incubator for 24 h. Medium may be changed after 4–6 h. Each experiment was performed six times. The transfected cells were detected by inverted fluorescence microscope (Olympus IX71), then digested by trypsin and adjusted to a cell concentration of 5×10^6 /mL. The transfection efficiency was detected using flow cytometry (Beckman).

Results

Separation of sc pDNA from oc pDNA

Two different size plasmids pCDNA3.1-GFP (7,200 bp) and pT-survivin 2B (3,500 bp) were used as model plasmids. Cells were lysed and clarified by conventional "alkaline lysis procedures". In order to further remove impurities from host cells in the

clarified lysates, a CaCl_2 precipitation step was taken before polishing.

A DEAE-Sepharose AEC was carried out for resolving proteins and nuclease contaminants and separating sc pDNA from oc pDNA. Two different size plasmids showed similar chromatogram in which there were a big flow-through peak and two elution peaks, a small but clear peak (first elution peak) followed by a big sharp peak (second elution peak), during the salt gradient elution process (Fig. 1). The results from agarose gel electrophoresis showed that the flow-through peak, first elution peak and second elution peak corresponded to non-nuclease impurities, oc pDNA and sc pDNA, respectively, and the yield of sc pDNA was $65 \pm 8\%$ (Fig. 2).

The quality of the purified pDNA was estimated by a series of analytics. The final preparations of pDNA had an A_{260}/A_{280} ratio of 1.93 ± 0.05 , which was regarded as high purity. The contaminants of genomic DNA and RNA were not detected in the agarose gel electrophoresis. The homogeneity, expressed as percentage of the sc isoform, of the obtained pDNA was estimated to be $95 \pm 1.1\%$ by gel scanning analysis. The protein contaminants were not detected by the BCA method. Endotoxins analysis revealed that the level of endotoxins in the preparations was lower than $0.003 \text{ EU } \mu\text{g}^{-1} \text{ pDNA}$, which well complied with the specification of $<0.1 \text{ EU } \mu\text{g}^{-1} \text{ pDNA}$ for gene therapy.

Biological activity detection

The results from in vitro transfection tests with 7,402 cells showed that the sc pDNA purified in the present work had higher transfection efficiency than the control pDNA, prepared with the OMEGA kit which is widely used for the small-scale preparation of pDNA for gene therapy in the world. The inverted fluorescence microscope analysis revealed that more cells were transfected by sc pDNA than by the control pDNA (Fig. 3), and the transfection efficiencies, estimated by flow cytometry analysis, of sc pDNA and control pDNA were $29 \pm 0.4\%$ and $20 \pm 0.1\%$, respectively (Fig. 4).

Discussions

For the production of pharmaceutical-grade pDNA at an industrial scale three main requirements have to be met. First, the product must be of high purity, free from bacterial genomic DNA, RNA, Protein, endotoxins. Second, the purified pDNA must be of high homogeneity, meaning most plasmid in supercoiled form. Last, the process must be cost-effective, scalable and easy to control quality.

Alkaline lysis procedures are routinely used in harvesting and partially purifying of plasmid. By this method, *E.coli* cells can be effectively broken up, and

Fig. 1 Chromatographic separation of pDNA isoforms with AEC. Linear gradient was performed at 7 mL/min by increasing the NaCl concentration in the eluent from 0.3 to

0.5 M in 10 mM Tris/HCl, pH 8.0. **a** pCDNA3.1-GFP (7,200 bp), **b** pT-survivin 2B (3,500 bp)

Fig. 2 Agarose gel electrophoresis analysis. **a** pCDNA3.1-GFP (7,200 bp). Lane 1 DNA ladder; Lane 2 lysate; Lane 3 flow-through; Lane 4–5 first peak; Lane 6–7 second peak. **b** pT-survivin 2B (3,500 bp). Lane 8 DNA ladder; Lane 9 lysate; Lane 10 flow-through; Lane 11 first peak; Lane 12 second peak

Fig. 3 Result of inverted fluorescence microscope ($\times 100$) **a** Plasmid purified using the OMEGA kit **b** Plasmid purified with the protocol of the present work

Fig. 4 Transfection efficiency detected by flow cytometry **a** negative **b** Plasmid purified with the protocol of the present work **c** Plasmid purified using the OMEGA kit

the majority of impurities from host cells such as genomic DNA, host protein, and RNA was precipitated and removed, but further purification and separation of sc pDNA from oc pDNA is essential to meet the requirements of pharmaceutical-grade

pDNA. Chromatography is easy to scale up and considered as the method with highest resolution, therefore being essential for producing pDNA for therapeutic applications. To remove trace contaminants and separate sc pDNA from oc pDNA, multiple

chromatographic steps are frequently employed. However, just one single AEC step was employed to achieve above goal in our novel process. It is reasonable to conclude that DEAE Sepharose Fast Flow used in the process is the ideal AEC medium for separation sc pDNA from oc pDNA, and a CaCl_2 precipitation step maybe have significant benefits for the subsequent purification steps.

The novel purification process for pDNA developed in present work met all above requirements for producing pharmaceutical-grad plasmid. Results from a series of analytics showed that the final purified pDNA contained extremely low amounts of genomic DNA, RNA, Protein and endotoxins, and had homogeneity higher than the specification of 90% (Schleef and Schmidt 2004). The biological activity of the purified pDNA was also testified to be significantly higher than that of the control pDNA by In vitro transfection.

The process developed in this work is cost-effective and scalable since just one single chromatographic step is employed, allowing trouble-free transfer from lab-scale preparation to large-scale production. Furthermore, it has significant advantages over traditional laboratory methods because the use of enzymes or animal-derived products is avoided, and there are no known or suspected toxic, mutagenic, carcinogenic, teratogenic, or otherwise harmful compounds used in the process.

Acknowledgments Supported by National Natural Science Foundation (30572124), Guangdong Natural Science Foundation (5002855), Guangdong Science and Technology Plan Project (2004B31201001) and Guangdong Pharmaceutical University doctor science starting foundation

References

- Bencina M, Podgornik A, Strancar A (2004) Characterization of methacrylate monoliths for purification of DNA molecules. *J Sep Sci* 27:801–810
- Cupillard L, Juillard V, Latour S, Colombet G, Cachet N, Richard S et al (2005) Impact of plasmid supercoiling on

- the efficacy of a rabies DNA vaccine to protect cats. *Vaccine* 23:1910–1916
- Diogo MM, Queiroz JA, Monteiro GA, Martins SA, Ferreira GN, Prazeres DMF (2000) Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnol Bioeng* 68:576–583
- Diogo MM, Ribeiro SC, Queiroz JA, Monteiro GA, Tordo N, Perrin P et al (2001) Production, purification and analysis of an experimental DNA vaccine against rabies. *J Gene Med* 3:577–584
- Diogo MM, Queiroz JA, Prazeres DMF (2003) Assessment of purity and quantification of plasmid DNA in process solutions using high-performance hydrophobic interaction chromatography. *J Chromatogr A* 998:109–117
- Diogo MM, Queiroz JA, Prazeres DMF (2005) Chromatography of plasmid DNA. *J Chromatogr A* 1069:3–22
- Ferreira GNM, Cabral JMS, Prazeres DMF (1997) A comparison of gel filtration chromatographic supports for plasmid purification. *Biotechnol Tech* 11:417–420
- Gurunathan S, Klinman DM, Seder RA (2000) DNA vaccines: immunology, application, and optimization. *Ann Rev Immunol* 18:927–974
- Hines RN, O'Connor KC, Vella G, Warren W (1992) Large-scale purification of plasmid DNA by anion-exchange high-performance liquid chromatography. *Biotechniques* 12:430–434
- Horn NA, Meek JA, Budahazi G, Marquet M (1995) Cancer gene therapy using plasmid DNA: purification of DNA for human clinical trials. *Hum Gene Ther* 6:565–573
- Iuliano S, Fisher JR, Chen M, Kelly WJ (2002) Rapid analysis of a plasmid by hydrophobic-interaction chromatography with a non-porous resin. *J Chromatogr A* 972:77–86
- Pillai VB, Hellerstein M, Yu T, Amara RR, Robinson HL (2008) Comparative studies on in vitro expression and in vivo immunogenicity of supercoiled and open circular forms of plasmid DNA vaccines. *Vaccine* 26:1136–1141
- Prazeres DMF, Schluep T, Cooney C (1998) Preparative purification of supercoiled plasmid DNA using anion-exchange chromatography. *J Chromatogr A* 806:31–45
- Sandberg LM, Bjurling A, Busson P, Vasi J, Lemmens R (2004) Thiophilic interaction chromatography for supercoiled plasmid DNA purification. *J Biotech* 109:193–199
- Schleef M, Schmidt T (2004) Animal-free production of ccc-supercoiled plasmids for research and clinical applications. *J Gene Med* 6:S45–S53
- Schluep T, Cooney CL (1998) Purification of plasmids by triplex affinity interaction. *Nucleic Acids Res* 26:4524–4528
- Wils P, Escriou V, Warnery A, Lacroix F, Lagneaux D, Ollivier M et al (1997) Efficient purification of plasmid DNA for gene transfer using triple-helix affinity chromatography. *Gene Ther* 4:323–330